A DISSERTATION ON

Extraction of Bioactive Peptides from *Rauvolfia serpentina*

SUBMITTED TO THE DEPARTMENT OF BIOSCIENCES

INTEGRAL UNIVERSITY, LUCKNOW

in partial fulfillment for the
Degree of Master of science in BiochemistryBy

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CERTIFICATE OF ORIGINAL WORK

This is to certify that the study conducted by **Mr. Suraj Yadav** during the months Feb-June, 2022 reported in the present thesis was under my Co-supervision. The results reported by his are genuine and script of the thesis has been written by the candidate himself. The thesis entitled is "**Extraction of bioactive peptides from *Rauvolfia serpentina***" is therefore, being forwarded for the acceptance in partial fulfillment of the requirements for the award of the degree of M. Sc Biochemistry, Department of Biosciences, Integral University, Lucknow, (U.P).

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TO WHOM IT MAY CONCERN

This is to certify that **Mr. Suraj Yadav**, a student of M. Sc. Biochemistry (II Year, IV semester), Integral University has completed his four months dissertation work entitled "**Extraction of bioactive peptides from *Rauvolfia serpentina***" successfully. he has completed this work from February of June 2022 at CSIR-CIMAP, under the guidance of **Dr. Prema G. Vasudev** The dissertation was a compulsory part of his M. Sc. degree.

I wish his good luck and future endeavors.

Dr. Snober S. Mir
Head,
Department of Biosciences,
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DECLERATION

I hereby declare that this project work titled "**Extraction of bioactive peptides from *Rauvolfia serpentina***" is a record of original work done by me under the supervision of **Dr.Prema G Vasudev, Principal Scientist,** Plant Biotechnology Division, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow and this project work has not formed on the basis for the award of any Degree/Diploma Association/Fellowship or similar title to any candidate of the Institute.

(Suraj Yadav)

Place: Lucknow
Signature with name

Date: Enrollment No:

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It is a matter of immense pleasure and pride for me to present my report entitled "Extraction of Bioactive Peptides from Rauvolfia serpentina*" which is an outcome of my training in the Plant Biotechnology Division, CSIR-CIMAP, Lucknow.*

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SURAJ YADAV

CONTENTS

INTRODUCTION

Plants are a rich source of metabolites like proteins, peptides, carbohydrates, alkaloids and flavonoids which play roles in plant growth & development and defences mechanism. Plant metabolites are categorised into two groups viz. primary metabolites and secondary metabolites. Primary metabolites are important for growth and development and are present in all plants, [1] whereas the secondary metabolites are present in specific organisms. Plant secondary metabolites are considered as evolutionary products that playan important role in the adaption of plants during biotic and abiotic stress [2, 3]. Plant proteins and peptides play major role in plant growth and development processes. Plant peptides are also involved in plant defence mechanism directly or indirectly. These peptides are generated from larger proteins by proteolytic processing Plant peptides can be categories into several families on the basis of their function and amino acid sequences [4]. These are a lipid-transfer proteins, thionin, havein, Snakin, defenis. Knottins, cyclotides, Mannose binding protein-1, vicilin like, and Imparitins, etc [5]. Biologically active peptides which are able to inhibit the growth of host pathogen, are called antimicrobial peptides (AMPs). Antimicrobial peptides are glycine or cysteine rich proteins with small in size and amphipathic nature. AMPs have valuable pharmacological properties such as antimicrobial, anticancer, cytotoxic, haemolytic, anti-HIV and insecticidal activity [6,7, 8, 9, 10]. Some of the insecticidal peptides from plants show binding affinity with the eukaryotic membrane [11, 12], which are useful in drug development as they possess the ability to penetrate the eukaryotic membranes. They can be used for developing transgenic pest-resistant varieties of agricultural crops [13, 14, 15]. Therapeutic peptide drugs obtained from peptides are preferred because of their low toxicity. [16]. Plant AMPs have been isolated from leaves, roots seeds, flowers and stems of many plant families.

Medicinal plants have a lot of pharmacological properties such as antimicrobial, anticancer, antidiabetics, cytotoxic, insecticides, etc. *R. serpentina* is a medicinal plant belonging to Apocynaceae family. The common names of *R. serpentina* are Sarpagandha, Snake root plant,

Chotachand, Chandrika, etc. It is an important medicinal plant in Ayurvedic and folklore medicine for the treatment of arthritis, skin cancer, eczema, burns, psoriasis, high blood pressure, digestive problems, sedative and diabetes [17, 18]. Major secondary metabolites from *R. serpentina* are indole alkaloids. They are found in all plant parts, but maximum in root bark. *R. serpentina*, which is used as natural tranquilizer, which Reserpineserpentine, papaverine, aricine, yohimbine, ajmalicine, deserpidine, sarpagine, thebaine, raubasine are some of thealkaloids used in treatment of breast cancer and hypertension [20, 21]. In this project extraction of bioactive peptides from the leaves of *Rauvolfia serpentina* was carried out.

REVIEW OF LITERATURE

Plant natural products are varied in structural and functional diversities. Many plants produce a specific type of compounds which is responsible for adaption of plant. Bioactive peptides are produced from larger precursor proteins by the selective action of endopeptidases which cleave at pairs of basic amino acids, usually Lys-Arg or Arg-Arg sites [22]. Charged residues appear to play an important role in pathogenic bacterium activity. Plant antimicrobial peptides contain around positively or negatively charged amino acids specifically arginine or lysine [23]. The main AMP families are defensins, thionins, α- hairpinins (hairpin-like peptides), hevein-like peptides, knottins, snakins, lipid transfer proteins, and cyclotides. Some peptides do not belong to these families, among them peptides with unusual Cys-motif, lacking disulphide bonds, cyclic peptides without cysteine knot and glycine, histidine-, alanine rich peptides [23]. According to the Data Repository of Antimicrobial Peptides (DRAMP), currently, more than 800 peptides have been annotated in plants [23]. AMPs are an important element of the innate immunity of plants, especially to biotic stress factors. AMPs have a wide spectrum of activities (antibacterial, antifungal, insecticidal, and antiviral), and some AMPs also inhibit hydrolases and protein biosynthesis. Due to their chemical properties, plants AMPs also demonstrate anti- proliferative action. **Table 1** shows a list of peptides reported from plants and their activities. *Rauvolfia serpentina* is an evergreen shrub belonging to the Apocynaceae family. The plant usually grows to a height between 60 and 90 cm and has pale green leaves that are 7 to 10 cm long and 3.5 to 5.0 cm wide. The leaves are elliptical or lanceolate shaped and occur inwhorls of 3 to 5 leaves. The plant has many shiny, black or purple, round fruits that are approximately 0.5 cm in diameter. It also has small pink or white flowers. The plant has a prominent tuberous, soft taproot that reaches a length between 30 and 50 cm and a diameter between 1.2 and 2.5 cm [24].

R. serpentina was used in folk medicine in India for centuries to treat a wide

variety of maladies, including snake and insect bites, febrile conditions, malaria, abdominal pain, and dysentery. It was also used as a uterine stimulant, febrifuge, and cure for insanity. The plant was mentioned in Indian manuscripts as long ago as 1000 BC and is also known as sarpagandhaand chandrika [25].

The genus *Rauvolfia* was named in honor of the 16th-century German physician Dr Leonhard Rauwolf, who studied plants while travelling in India. The name *Serpentina* was selected for study due to its long, tapering, snake-like roots. The Indian political leader Mahatma Gandhi was known to employ *Rauvolfia*, reportedly using the root to make a tea that he consumed in the evening to help relax after a busy, overstimulated day. The Indian physician Rustom Jal Vakil is considered responsible for introducing *Rauvolfia*Western medicine. He collected data on patients treated with *Rauvolfia* for 10 years, from 1939 to 1949. In 1949, he published a watershed paper on the antihypertensive properties of *R. serpentina* in the *British Medical Journal* [24]. He presented his detailed results from treating 50 patients who had high blood pressure with the root of *Rauvolfia*. The results were remarkable and significant. By 1949, more than 90% of Indian physicians were using *Rauvolfia* in the treatment of high blood pressure. After Vakil's original paper, more than 100 scientific articles were published throughout the world [24].

Classification:

Kingdom: Plantae Subphylum: Angiospermae Class: Dicotyledonae Family: Apocynaceae Genus: Rauvolfia
Species: *Rauvolfia serpentina*

Figure:1 *Rauvolfia serpentina*

Distribution:

More than 100 species are included in the *Rauvolfia* genus, and they are native to tropical and subtropical regions of the world, including Europe, Africa, Asia, Australia, and the Central and South Americas. *Rauvolfia serpentina* is native to the moist, deciduous forests of southeast Asia, including India, Burma, Bangladesh, Sri Lanka, and Malaysia [27].

Chemical Composition:

Numerous different phytochemicals, such as alcohols, sugars, glycosides, fatty acids, flavonoids, phytosterols, oleoresins, steroids, tannins, and alkaloids are all present in *Rauvolfia* plant. More than 50 of the plant's indole alkaloids have been isolated and making them the most significant alkaloids present [26]. A class of nitrogenous substances called indole alkaloids is derived from the amino acid tryptophan [27]. They have a single nitrogen molecule and a heterocyclic ring structure with five and six carbons in common.

Indole alkaloids are present in all plant parts, including the stem and leaves, but they are most abundant in the bark of the root. Included among the known indoles and indole alkaloids are *ajmalidine, ajmaline, ajmalinine, aricine, canescine, coryanthine, deserpidine, isoajmaline, isoserine, isoserpiline, lankanescine, neoajmaline, papaverine, raubasine, raucaffricine,* rauhimbine, *rauwolfinine, recanescine, rescinnamine, reserpiline, reserpine, reserpinine, sarpagine, serpentixne, serpentinine, thebaine, yohimbine, and yohimbinine*[28].

Medical Uses:

For the treatment of epilepsy and seizures, schizophrenia and bipolar disorder, as well as insomnia and sleep issues, *Rauvolfia* has been investigated. According to one study, *Rauvolfia* is useful for treating anxiety.

In that study, the whole root, alseroxylon, and reserpine were all used as forms of *Rauvolfia,* and they all had the same effects on ambulatory patients' overt anxiety. Between the ages of 3.5 and 9 years*, Rauvolfia* has been investigated as a potential in treating delirium tremens in patients who are alcohol and drug dependent, according to another study. A decrease in agitation, excitement, and treated migraines, resulting in an increase in quality of life and a decrease in pain. In another study, *Rauvolfia* was used to treat angina pectoris in patients with coronary artery disease. The study found that the angina symptoms decreased and the therapeutic effect lasted longer. In that study, fifty percent of the patients went on to have electrocardiograms that were normal. In a different study, *Rauvolfia* was investigated to see if it could treat pruritic and psychogenic dermatoses. Additionally, it is said to reduce psoriatic outbreaks.

Table 1: Some peptides from plants and their activities:

Sr.no	**Name of peptide**	**Source of peptide**	**Biological activities**	**References**
1	JS1JS2	*Jasminumsamba c(L.)*	CytotoxicityAssay Hemolysis Assay Antimicrobial Activity Carboxypeptidase Inhibition Assay	29
2	bevuTI-I	*Beta vulgaris*	Trypsin Inhibition Assays.Prolyl Oligopeptidase Inhibition Assays	30
3	hylin a-1hylin b-1hylinb-2	*Hypsiboas albopunctatus Hyl abiobeba*	Antimicrobial activity Haemolysis activity	31
4	Atr-SN1 Atr-DEF1 Atr-LTP1	*Amaranthus tricolor*	Antimicrobial activity	32

5	CC-AMP1CC-AMP2	*Capsicum annuum* (hot peppers)	ESKAPE Pathogen Assays Outer Membrane Permeabilization Assay	33
6	ribifolin	*Jatropha ribifolia*	Antiplasmodial Activity	34
7	RCB-1 RCB-2 RCB-3	*Ricinus communis*	Antimicrobial agents Cytotoxic Activity	35
8	PepSAVI-MS	*Maranthus tricolor*	Antimicrobial activity	36
9	B, cyclo-, C, cyclo-	*Viola arvensis*	Antimicrobial activity	37
10	oscillamide C	*Lanktothrix* (Oscillatoria) *agardhii* and *P. rubescens.*	protein Phosphatase Inhibition Assay	38
11	Poca a Poca b	*Pombalia calceolaria*	Wound-Healing and Cytotoxicity Assay.	39
12	Cycloviolacin	*Viola odorata*	Larval Bioassay	40
13	Planktocyclin,	*Planktothrix rubescens*	Protease Inhibition Assays	41

AIM AND OBJECTIVES

Aim: Extraction of Bioactive Peptides from *Rauvolfia serpentina*

Objectives:

Collection of Plant material from CIMAP Farm facility Lucknow. Extraction of peptide from plant part using aqueous buffer. Purification of peptide from the crude extract.

MATERIALS AND METHODS

Materials:

Chemicals and Plasticwares	**Manufacturer**
Acetic acid	Merck
Methanol	Merck
Pyridine	Merck
Ninhydrin reagent	Alfa assar
Tris Buffer [Tris (hydoxymethyl) Aminomethane]	Sigma-Aldrich
Sodium Chloride	HiMedia Laboratory Pvt. Ltd
Sodium dodecyl sulphate (SDS	Sigma-Aldrich
Coomassie Brilliant Blue G-250	Sigma-Aldrich
Tricine buffer [Tris (hydroxymethyl) methylglycine]	Sigma-Aldrich
TEMED (Tetramethylethylenediamine)	HiMedia Laboratory Pvt. Ltd
Acrylamide	Sigma-Aldrich
Bis-acrylamide	Sigma-Aldrich
Ammonium Persulphate (APS)	Merck
Bovine serum albumin (BSA)	HiMedia Laboratory Pvt. Ltd
Bradford reagent	HiMedia Laboratory Pvt. Ltd
Sodium dihydrogen phosphate	Merck Life sciences
Disodium hydrogen phosphate	Merck life sciences
Ammonium sulphate	Sissco Research Laboratory
1N HCl	Merck Life sciences
Protein ladder (1.3-26.6kDa)	Sigma-Aldrich
Silica Gel plate 60 (TLC plate)	Merck
n-Butanol	Merck
Syringe filter (0.45µm)	Genexy
Amicon centrifugal devices (MW cut-off membrane)	Merck Millipore
Skanin Dialysis membrane	Thermofisher Scientific

Instruments:

Instruments	**Company**
Bench-top Centrifuge	Eppendorf
Gel Electrophoresis apparatus	Biochem
Spectrophotometer	Thermofisher scientific
Gel Doc for Proteins	UVP
Refrigerator (4ºC and 20ºC)	Samsung
Weighing balance	Metller Teldo

METHODOLOGY

Collection of mature leaves from *Rauvolfia serpentina*:

The plant leaves (30.0g) were collected from pots which are grown CSIR-CIMAP Lucknow. All leaves are fresh and matured. The leaves were washed sequentially with tap water, distilled Water, MQ water and 70% Ethanol and dried it using blotting paper. The dried leaves were crushed in liquid nitrogen using pistol & mortar to make fine powder and stored at -80ºC for further experiments.

Extraction of bioactive peptides:

grams of leaf powder was weighed and was suspended in 250 ml of 25mM phosphate buffer (pH 7.0), incubated at 4ºC for 12-14 hours, on magnetic stirrer at 200 rpm. The extract was centrifuged at 5000 rpm for 10 min. at 4ºC. The supernatant (140 ml) was collected and filtered using 0.45µm membrane filter. Stored at 4ºC for further experiments Purification of peptides from the crude extract:

Protein precipitation using ammonium sulphate

Protein precipitation was done different concentration of ammonium sulphate. 16.24 g of solid ammonium sulphate was slowly added into 70 ml of plant crude extract until the concentration reached 40% and kept at 4ºC for 6 hours. Saturated solution was centrifuge at 10000 rpm for 10 min. at 4ºC. Collected the pellets and supernatant. Next 80% ammonium sulphate saturation was done by adding 20.94 g of solid ammonium sulphate in 78 ml of 40% ammonium sulphate saturated solution and kept at 4ºC for 6 hours. Centrifuge at 10000 rpm for 10 min. 4ºC. Collected the pellets and dissolved in 2 ml of 20mM Tris-Cl pH 8.0.

Dialysis of total soluble protein

The total soluble protein was dialysed against 300 volume of 20mM Tris-Cl pH 8.0 using 3.5kDa MW cut-off dialysis membrane at 4ºC for 4-6 hour.

Desalted protein solution was concentrate using centrifugal devices (3kDa MW cut-off membrane, Amicon ultra-4)) at 4500 rpm, 4ºC. Check the protein purity and protein concentration using Tricine SDS-PAGE and Lowry Assay.

Fractionation of desalted protein with different molecular weight cut-off membrane:

The desalted proteins were (30kDa, 10kDa and 3kDa, Amicon-ultra). 2 ml of desalted protein solution was loaded into 30kDa MW cut-off membrane centrifugal device, centrifuge at 4500 rpm for 15 min. at 4ºC. Collected the permeated fraction and loaded into 10kDa MW cut-off membrane centrifugal device, centrifuged and collected the concentrate and permeate fractions. The 10kDa permeated fraction was loaded into 3kDa MW cut-off membrane, centrifuged and collected the concentrate and permeate fractions. Stored at 4ºC for further experiments.

SDS-PAGE electrophoresis:

All fraction were analyzed using tricine SDS-PAGE. Tricine SDS-PAGE is used for separation of low-molecular-mass protein according to the molecular weight [42]. Proteins are separated out based on charge to mass ratio. Preparation of resolving gel and stacking gel, buffers, loading dyes for the experiment is provided in Annexure. The resolving gel components were mixed and were immediately poured into the gel plate assembly. The top of the resolving gel was layered with Isopropanol, to remove traces of air bubble and also make the smooth surface of resolving gel. After solidification of resolving gel, the isopropanol/water layer was removed and the gel was washed with water. The stacking gel components were mixed in a vial andpoured above the resolving gel. 10 wells weremade by placing a comb inside the stacking gel. The gel plate assembly was transferred to the gel unit containing cathode and anode buffers. Peptides fractions were mixed with 2X loading dye in a micro centrifuge tube, incubated at 93ºC for 10 minutes on a water bath. 20µl of denatured sample were loaded into the wells. A low molecular weight polypeptide marker from Sigma-Aldrich (1.4kDa to 26.6kDa MW range) was also loaded as reference. The gel was then run by applying constant voltage (90 V). After the dye reached the bottom of the gel casket; the gel was carefully transferred into a polypeptide fixative solution (Annexure) for 25-30 minutes. The gel was then transferred to a box

containing Coomassie Brilliant Blue G-250 (CBB G-250) stain solution (Annexure) and was kept on a shaker at room temperature for 4hours to overnight. After the gel was uniformly stained, the staining solution was poured off and the destaining solution (Annexure) was added. Destaining step was repeated 3-4 times until the bands were clearlyvisible.

Peptides detection by Ninhydrin Test:

Ninhydrin spray reagents are widely used to analyse and characterize amino acids, peptides, and proteins. The mechanism of the reaction of ninhydrin hydrate with α- amino acid of peptides, producing the colored ninhydrin chromophore called Ruhemann's purple (RP) [43]. Ninhydrin test of peptides detection was done by Thin Layer Chromatography (TLC). The Peptides rich fractions were spotted on TLC plate (Silica Gel 60, 5x20cm) by capillary tube, dried for few minutes at room temperature. The TLC plate was kept in 100 ml of developing solution (n-Butanol/Acetic acid/ Water, 3:1:1) for 3-4 hours to migrate of peptides at different retention factor. After run the solvent, sprayed the 1% Ninhydrin solution (1% Ninhydrin in Pyridine/glacial acetic acid, 5:1) and heat at 100º C for 5 min. After heat incubation, appeared the purple spot-on TLC plates and analysed with known compound [43].

RESULTS AND DISSCUSION

Aqueous buffer solutions are the most common extractant for the isolation of plant peptides such as antimicrobial peptides, Angiotensin-I-Converting Enzyme (ACE)-Inhibitory peptides. Phosphate buffer is the most used extractant for plant peptides isolation [44, 45, 46, 47]. The pH value used is close to neutral (7.4-7.9). Using the phosphate buffer solutions as extractant leads to the extraction of bioactive peptides from *Rauvolfia* leaves, extraction buffer was taken 10 times of plant tissues powder. The plant crude extract was 140 ml. The next step after extraction was protein precipitation and purification. Protein precipitation was done by ammonium sulphate saturation method and purification was done by molecular cut-off membrane. Protein precipitation and purification was done at 4ºC. After protein precipitate and dialysis, Total soluble protein (TSP) concentration is 3.86 mg/ml which is quantified by Lowry assay. The dialysed TSP was fractionation different MW cut-off membrane. The protein concentration of different fractions is 1.08 mg/ml (30kDa MW cut-off fraction), 0.16mg/ml (10kDa MW cut-off fraction) and 0.06 mg/ml (3kDa MW cut-off fraction). All fractions were analysed the molecular weight of peptides using 16.5% Tricine SDS-PAGE and gel staining with CBB-G250.

After extraction and purification of peptide, quantify the concentration of peptides rich fractions estimated by Lowry method.

Table1: Absorbance of BSA standard at different protein concentrations

Concentration of standard (mg/ml)	**Absorbance at 660 nm**
0	0.000
1	0.058
2	0.098
3	0.121
4	0.161
5	0.224
6	0.293

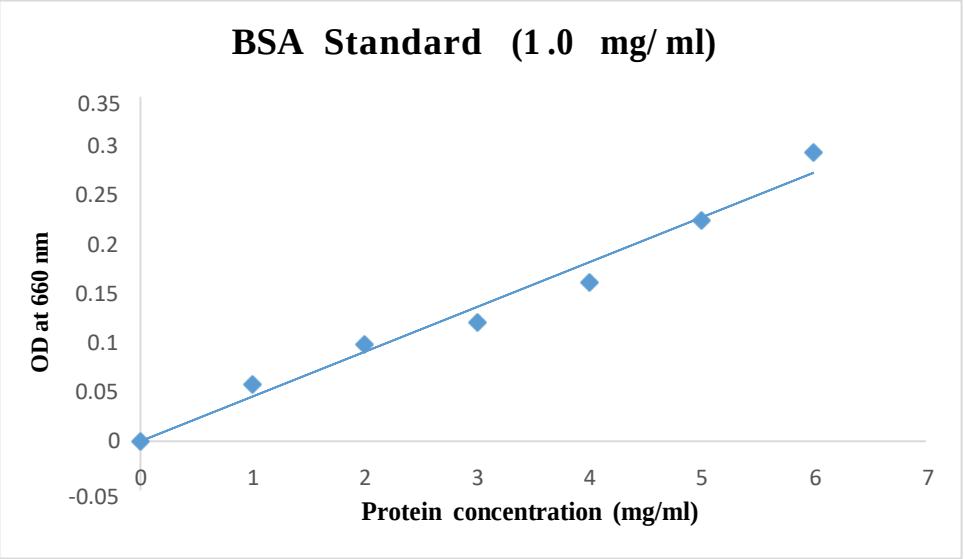
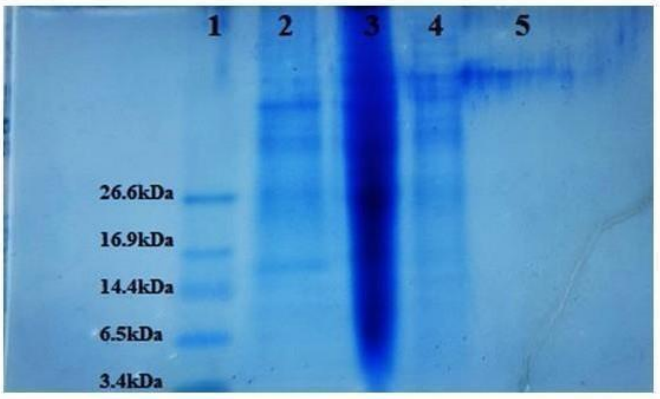


Figure 2 : Linear graph of concentration of protein Vs OD at 660 nm.

Table 2: Protein concentration of sample

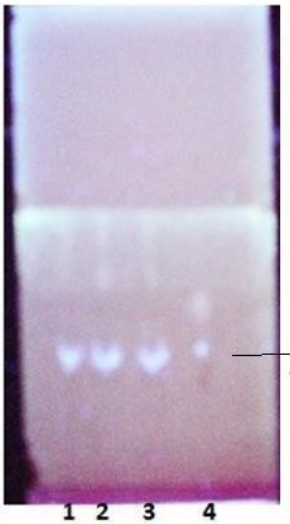
Samples	**Protein concentration (mg/ml)**
Crude extract	4.23
30kDa MW fraction	1.19
10kDa MW fraction	0.18
3kDa MW fraction	0.06



Lane 1: Proteins marker (1.6-26.6kDa), 2: Crude extract, 3: 30kDa Rentent fractions, 4: 10kDa Rentent fractions, 5: 3kDa Rentent fractions

Figure 3: 16.5% Tricine SDS-PAGE of peptide rich fractions

Silica gel column chromatography (TLC Silica gel 60) was performed on the crude extract as well as molecular weight fractions (10kDa MW cut-off fractions, 3kDa MW cut-off fraction and 3kDa permeated fraction). Ninhydrin reagent is used for detection of peptides in the solution. Ninhydrin binds with free amino group of peptides or protein to developed the purple colour. In this fraction, no shown any purple colour in visible range but one spot shown in UV range. So, peptides or proteins are also detected in UV range.



Lane 1: 10kDa Rentent fraction, Lane 2: 3kDa Rentent fraction, Lane 3: kDa permeated fraction, Lane 4: standard (Peptides mixture)

Figure 4: Thin Layer Chromatography (TLC) of peptide rich fractions

CONCLUSION

The leaves of *Rauvolfia serpentina* plant were extracted using 25mM of phosphate buffer and examined for the presence of low molecular weight polypeptide in Tricine SDS- PAGE. It was found that the leave of *R. serpentina* contain polypeptides of high molecular weight of 30 KDa. Very few protein bands were observed for polypeptides with 10KDa and below. Due to very low protein concentration they were not further purified.

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APPENDIX

Lowry Assay:

Lowry assay is performed for estimating of the protein concentration in the sample by takingBSA as a standard in different concentrations.

Preparation of BSA solution as Standards:

Reagents:

The Bovine Serum Albumin (BSA) was taken as standard and was prepared by dissolving 1 mg BSA in 1 ml of distilled water.

Lowry's reagent:

10 ml of 2%Na_2CO_3mixed with o.1N NaOH (40 mg

in 10 ml H_20). 10 ml of 1% $CuSO_4$.

2% sodium potassium tartrate solution mixed in distilled water. 3.Folin Coicalteu reagent (2N) FC Reagent

4. Mixed and prepared Lowry solution in ratio 48:1:1 (A+B+C)

Added 1.0 ml of Lowry reagent to the samples and incubated for the 10 min at room temperature.

Added 100µl F-C reagent and mixed gently kept at room temperature darkness for 30min Taken the absorbance at 660 nm in UV Spectrophotometer.

Plotted the graph against protein concentration vs

absorbance Tricine SDS-PAGE for Polypeptides:

Preparation of SDS-

PAGE Reagents: 30%

acrylamide bisacrylamide

solution.

29 g of acrylamide and 1gm his-acrylamide was dissolved in a total volume of 60 ml distilled water. The solution was then heated to 37 °C. The volume was adjusted to 100 ml with distilled water.

Tris CI/SDS (3M Tris-CI, 0.3 % SDS, pH 8.45):

182 g of Tris base was dissolved in 300 ml o^{2}f^{9}distilled water and adjusted the pH to

8.45 with HCl. The volume was made up to 500 ml by adding distilled water. 1.5 g of SDS was added and stored at 4 °C.

10% (w/v) Ammonium persulphate:

0.1g of APS was dissolved in 1 ml of distilled water and stored at 4 °C.

Gel Running Regents:

5X Cathod buffer (0.1 M Tris, 0.1 M Tricine and 0.1% SDS): Added 12.11 g of Tris base, 17.92 g of Tricine and 1g of SDS in 1 liter of dist. Water. Stored at 4ºC.

5X Anode buffer (0.2 M Tris Cl, pH 8.9): Added 24.22 g of Tris base in 1 liter of dist. Water. Stored at 4ºC.

2X Tris-Tricine Sample Buffer:

Distilled water	4.0 ml
0.5M Tris-Cl pH 6.8	2.0 ml
Glycerol	2.4 ml
10% SDS	1.0 ml
ß-Mercptoethanol	0.2 ml
0.05%(w/v) CBB G-250	0.4 ml

Polypeptide fixation solution: Methanol/ glacial acetic acid/water (4:1:5)

0.25%(w/v) Coomassie Blue G-250 staining solution: 0.25 g of CBBG-250 was dissolved in10% glacial acetic acid/water. Stored at RT.

Coomassie Blue G-250 destaining solution: 10% (v/v) acetic acid (10 ml of glacial aceticacid in 90ml of dist. Water. Stored at RT.

Table 2: Tricine SDS-PAGE gel recipes:

Stock solution	**16.5% Separating gel**	**4% Staking gel**
29:1 acrylamide/Bis-acrylamide	2.17 ml	0.48 ml
3M Tris Cl/SDS. pH 8.45	2.00 ml	1.24 ml
Distilled water	1.19 ml	3.47 ml
24% (w/v) Glycerol	0.63 ml	

10% APS	0.020 ml	0.010 ml
TEMED	0.004 ml	0.004 ml

Sample preparation:

20 µl of the sample was mixed with 10 µl of 2X Tris-Tricine sample buffer and was incubated at 95 °C for 10 min. The samples were then spun at 5000 rpm for 5 min. Dilution of the polypeptide marker: Diluted Standards (polypeptide marker) 1µl polypeptide solution was mixed in 19µlof 2X Tris -Tricine sample buffer and incubated for 5 minutes at 95 °C.

Loading of polypeptide marker and samples:

The polymerized gel was mounted in the vertical electrophoretic apparatus. Anode buffer was poured into the gel apparatus tray and cathode buffer was poured on thetop into the wells and comb was removed.

10 µl of diluted polypeptide marker was cooled and loaded for mini gel. The 20 µl of each sample were cooled and loaded in the wells.

Electrophoretic conditions:

The running conditions were set to 40V voltage for staking gel and

90V voltage for separating gel. The gel was allowed to run when the

dye front runs out of the gel. Gel staining and destaining:

The gel was placed in polypeptide fixative solution for 30 min. Staining was done by keeping the gel in Coomassie Blue G- 250 staining solution for 1 hr. The gel was destained by keeping in Coomassie Blue G- 250 destaining solution for 3times for 15 minutes or overnight.

Protein bands Analysis: After destaining the gel was analysed the band under Gel Doc.